



Application Serial No.: 10/526,026

Filing Date: February 28, 2005

Assignee: ARKRAY, Inc.

Title of the Invention: METHOD FOR PURIFYING PROTEIN AND GLUCOSE  
DEHYDROGENASE

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Dated December 13, 2006

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【Name of Document】	Patent Application
【Reference Number】	P14-293830
【Filing Date】	August 30, 2002
【Receiving Office】	Commissioner of Patent
Office	
【IPC】	C07K 3/20
	C12N 15/53
【Title of the Invention】	METHOD FOR PURIFYING
PROTEIN AND GLUCOSE DEHYDROGENASE	
【Number of Claims】	14
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**【Deposit Number】** 024198

**【Amount of Payment】** 21000

**【Attached Documents】**

**【Name of Document】** Specification 1

**【Name of Document】** Drawings 1

**【Name of Document】** Abstract 1

**【Identification of**

General Power of

Attorney】 0103432

**【Proof】** necessary

【Identification of the Document】 SPECIFICATION  
【Title of the Invention】 METHOD FOR PURIFYING  
PROTEIN AND GLUCOSE DEHYDROGENASE

【Claims】

【Claim 1】

A method for purifying a target protein from a protein solution containing the target protein including electron transfer protein by using liquid chromatography, wherein the liquid chromatography is performed by introducing the protein solution into a column filled with a packing agent, causing the packing agent to hold the target protein, removing impurities, eluting the target protein in the packing agent by using an eluent containing a hydroxy-cholate.

【Claim 2】

The method for purifying protein according to Claim 1, wherein the target protein is provided by a glucose dehydrogenase containing a protein having a subunit including glucose dehydrogenation activity.

【Claim 3】

The method for purifying protein according to Claim 1 or 2, wherein the packing agent is provided by an ion-exchange resin.

【Claim 4】

The method for purifying protein according to Claim 3, wherein the ion-exchange resin contains a quaternary ammonium group as an ion-exchange group.

**【Claim 5】**

The method for purifying protein according to any one of Claims 2-4, wherein the electron transfer protein has a molecule weight of approximately 43 kDa in SDS-gel electrophoresis under a reducing environment, the subunit having a molecule weight of approximately 60 kDa in SDS-gel electrophoresis under a reducing environment.

**【Claim 6】**

The method for purifying protein according to any one of Claims 1-5, wherein the hydroxy-cholate comprises a cholate.

**【Claim 7】**

The method for purifying protein according to any one of Claims 1-6, wherein the hydroxy-cholate in the eluent is maintained at a constant concentration in the elution of the target protein from the packing agent.

**【Claim 8】**

The method for purifying protein according to Claim 7, wherein the concentration of the

hydroxy-cholate in the eluent is selected from a range of 0.5 through 2.5 wt%.

**【Claim 9】**

The method for purifying protein according to any one of Claims 2-8, wherein the glucose dehydrogenase is produced by a microorganism belonging to the genus *Burkholderia* which is capable of producing the glucose dehydrogenase.

**【Claim 10】**

The method for purifying protein according to Claim 9, wherein the microorganism belonging to the genus *Burkholderia* is provided by *Burkholderia cepacia* KS1 strain (FERM BP-7306).

**【Claim 11】**

The method for purifying protein according to any one of Claims 2-8, wherein the glucose dehydrogenase is produced by a transformant, wherein the transformant is produced by engineering a host microorganism with a DNA from a microorganism belonging to the genus *Burkholderia* which is capable of producing the glucose dehydrogenase for coding the subunit and the electron transfer protein.

**【Claim 12】**

The method for purifying protein according to Claim 11, wherein the host microorganism is provided by *Pseudomonas putida*.

**【Claim 13】**

A method for purifying glucose dehydrogenase using a combination of hydrophobic chromatography and anion exchange chromatography, wherein the hydrophobic chromatography includes: a step of causing a stationary phase to hold the glucose dehydrogenase; a step of eluting unnecessary proteins; and a step of eluting the glucose dehydrogenase by using an eluent containing a hydroxy-cholate, wherein the anion exchange chromatography includes: a step of causing a stationary phase to hold the glucose dehydrogenase; and a step of eluting the glucose dehydrogenase by using an eluent containing a hydroxy-cholate.

**【Claim 14】**

The method for purifying glucose dehydrogenase according to Claim 13, wherein concentration of the hydroxy-cholate in eluting the glucose dehydrogenase is varied with time in the hydrophobic chromatography, wherein concentration of the hydroxy-cholate in eluting the glucose dehydrogenase being kept at a constant level in the elution of the glucose dehydrogenase in the anion exchange chromatography.



## **【Detailed Description of the Invention】**

### **【0001】**

### **【Field of the Invention】**

The present invention relates to a method for purifying protein by using liquid chromatography. The method is used, for example, when purifying glucose dehydrogenase which is bonded to electron transfer protein.

### **【0002】**

### **【Prior Art】**

Development efforts have been made in many different fields of industry for biosensors incorporating an enzyme which makes a specific reaction with a specific substrate. A representative example of such biosensors is a glucose sensor utilized mainly in the field of medical care.

### **【0003】**

The glucose sensor establishes a reaction system which includes an enzyme and an electron transfer material. When using the glucose sensor, an amperometric method for example is employed to quantitate glucose. The enzyme is provided by e.g. glucose oxidase (GOD) and glucose dehydrogenase (GDH).

### **【0004】**

GOD has high substrate specificity to glucose, high thermal stability, and is less expensive than other enzymes because it can be mass-produced industrially. A shortcoming on the other hand is that reaction systems involving GOD are highly sensitive to oxygen dissolved in the sample, and so the dissolved oxygen can affect the measurement.

**【0005】**

On the other hand, reaction systems involving GDH are not susceptible to dissolved oxygen in the sample. For this reason, reaction systems which utilize GDH allow accurate measurement of the glucose level even if the measurement is made in an environment poor in oxygen partial pressure, or if the measurement is made to a high-concentration sample which has a high oxygen demand. Shortcomings of GDH include poor thermal stability and lower substrate specificity than GOD.

**【0006】**

Under these circumstances, efforts have been made in search for an enzyme which covers the shortcomings of both GOD and GDH. As disclosed in the International Disclosure W002/36779, Sode separated a new strain (Burkholderia cepacia KS1) from soil near a hot spring, and

obtained a new GDH from the strain. This GDH included  $\alpha$ ,  $\beta$ ,  $\gamma$  subunits (hereinafter called "CyGDH"), had a high rate of reaction with electron transfer materials, and sufficient thermal stability, and so was suitable for use in glucose sensors.

【0007】

When utilizing CyGDH in glucose sensors, CyGDH must be purified from an enzyme solution which contains CyGDH. Enzyme is usually purified by means of liquid chromatography, so the inventor et al. of the present invention followed a common method of hydrophobic chromatography and anion exchange chromatography in an attempt to process the enzyme solution. However, it was not possible to purify CyGDH to a high level of purification, as SDS-PAGE examinations revealed that the obtained enzyme solution contained a number of different proteins in addition to  $\alpha$ ,  $\beta$ ,  $\gamma$  subunits.

【0008】

【Means for Solving the Problems】

The present invention aims at providing a new method for purifying a protein.

【0009】

A first aspect of the present invention provides a method for purifying a target

protein from a protein solution which contains the target protein including electron transfer protein by using liquid chromatography. The liquid chromatography is performed by introducing the protein solution into a column which is filled with a packing agent, causing the packing agent to hold the target protein, removing impurities, and eluting the target protein in the packing agent by using an eluent which contains a hydroxy-cholate.

**【0010】**

It should be noted here that in the present specification, that the term "liquid chromatography" refers, unless otherwise specified, to both of a column method in which the purification is made in a flow (continuously), and a batch method of purifying protein. An example of the batch method is placing a packing agent and the protein solution together in a column to cause the packing agent to bind the target protein, then separating the packing agent, and then bringing the packing agent in contact with the eluent thereby separating and collecting the target agent from the packing agent.

**【0011】**

An example of the target protein, which is the object of the purification, is glucose dehydrogenase which contains a subunit including glucose dehydrogenation activity.

【0012】

The packing agent can be an ion-exchange resin, and the protein is purified by means of an ion-exchange chromatography. In this case, the ion-exchange resin contains a quaternary ammonium group as an ion-exchange group.

【0013】

Here, the term "hydroxy-cholate" refers to salts of cholic acid which are trihydrates of cholanic acids, as well as their derivatives in a broad sense. Examples of hydroxy-cholates include cholates, glicoursodeoxycholate, tauroglicoursodeoxycholate, tauroursodeoxycholate, ursodeoxycholate, glycocholate, taurocholate, glycochenodeoxycholate, taurochenodeoxycholate, glycodeoxycholate, taurodeoxycholate, chenodeoxycholate, deoxycholate, glycolithocholate, tauroolithocholate, and lithocholate. Among others, it is preferable to use a cholate such as sodium cholate.

【0014】

When eluting the target protein from the packing agent, preferably, the concentration of the hydroxy-cholate in the eluent is maintained at a constant level. In this case, the concentration of the hydroxy-cholate in the eluent is preferably selected from a range of 0.5 through 2.5 wt%, and more preferably, from 0.8 through 1.2 wt%. Such a method is applied not only to the elution of the target protein in a batch process but also to the elution of the target protein in a continuous process. Alternatively, the concentration of the hydroxy-cholate in the eluent may be varied with time, in eluting the target protein. In this case, the upper limit concentration of the eluting agent should be not higher than 3 wt% for example, and more preferably not higher than 1.5 wt%, and most preferably not higher than 1 wt%. In other words, the concentration of the eluent is varied within the range of 0-3 wt% for example, more preferably within the range of 0-1.5 wt%, and most preferably within the range of 0-1.0 wt%.

#### 【0015】

The electron transfer protein has a molecule weight of e.g. approximately 43 kDa in SDS-gel electrophoresis under a reducing environment.

On the other hand, the subunit has a molecule weight of approximately 60 kDa in SDS-gel electrophoresis under a reducing environment. Glucose dehydrogenase which contains such electron transfer protein and subunits is obtainable from e.g. a microorganism belonging to the genus Burkholderia which is capable of producing the glucose dehydrogenase, or from a transformant thereof.

**【0016】**

There is no specific limitation to the microorganism of the genus Burkholderia which is used in the present invention, as long as the microorganism is capable of producing the target enzyme. Preferably, however, Burkholderia cepacia, and Burkholderia cepacia KS1 strain (hereinafter simply called "KS1 strain") in particular, is preferred. KS1 strain was deposited as FERM BP-7306, on September 25th, 2000, with the International Patent Organism Depositary (IPOD), National Institute of Advanced Industrial Science and Technology (AIST) (Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan 305-8566).

**【0017】**

The transformant can be produced, for example, by engineering a host microorganism with a DNA

from a microorganism belonging to the genus Burkholderia for coding the electron transfer protein and the subunit. The host microbe is preferably a microorganism belonging to the genus Pseudomonas, Pseudomonas putida in particular, or E. coli bacterium.

**【0018】**

As disclosed in International Publication WO02/36779 and others, glucose dehydrogenase derived from KS1 strain contains a subunit ( $\alpha$  subunit) and an electron transfer protein ( $\beta$  subunit), and  $\gamma$  subunit which has a molecular weight of approximately 14 kDa in the SDS gel-electrophoretic migration under reducing environment.

**【0019】**

Sode has confirmed that higher enzyme activity is achieved by a combination of  $\gamma$  subunit and  $\alpha$  subunit than by  $\alpha$  subunit only. Therefore, in view of enzyme activity, it is preferable to manifest  $\gamma$  subunit, and when engineering the DNA,  $\gamma$  subunit structural gene should preferably be included in an upstream region of  $\alpha$  subunit. Then, when the transformant produces  $\alpha$  subunit,  $\gamma$  subunit which has been manifested already and existing as a protein



will promote efficient production of  $\alpha$  subunit in the microorganism.

**【0020】**

A second aspect of the present invention provides a method for purifying glucose dehydrogenase using a combination of hydrophobic chromatography and anion exchange chromatography. The hydrophobic chromatography includes: a step of causing a stationary phase to hold the glucose dehydrogenase; a step of eluting unnecessary proteins; and a step of eluting the glucose dehydrogenase by using an eluent containing a hydroxy-cholate. The anion exchange chromatography includes: a step of causing a stationary phase to hold the glucose dehydrogenase; and a step of eluting the glucose dehydrogenase by using an eluent containing a hydroxy-cholate.

**【0021】**

In the hydrophobic chromatography, elution of the glucose dehydrogenase for example, is performed by varying the concentration of the hydroxy-cholate in the eluent with time. On the other hand, in the anion exchange chromatography, elution of the glucose dehydrogenase for example, is performed by keeping the concentration of the hydroxy-

cholate in the eluent. Alternatively, the concentration of the hydroxy-cholate in the eluent may be varied with time, in eluting the target protein. In this case, the upper limit concentration of the eluting agent should be not higher than 3 wt% for example, and more preferably not higher than 1.5 wt%, and most preferably not higher than 1 wt%. In other words, the concentration of the eluent is varied within the range of 0-3 wt% for example, more preferably within the range of 0-1.5 wt%, and most preferably within the range of 0-1.0 wt%.

**【0022】**

**【Mode for Carrying out the Invention】**

Hereinafter, methods of purifying a protein according to the present invention will be described specifically, taking an example of purifying glucose dehydrogenase.

**【0023】**

When purifying glucose dehydrogenase, first, an enzyme solution is prepared. The enzyme solution may be obtained from a microbe which produces the glucose dehydrogenase, a culture of the microbe, a transformant derived through insertion of DNA which is extracted from the microbe, or a culture of the transformant.

【0024】

If GDH is inside the microbe, the enzyme solution can be obtained by first separating the microbe from the culture through filtration, centrifugation, etc., and then fragmentizing the microbe mechanically or enzymatically with e.g. lysozyme, followed as necessary, by solubilization of GDH through addition of a chelating agent such as EDTA and a surfactant. On the other hand, if GDH is outside the microbe (in the culture), the enzyme solution can be obtained by separating the microbe from the culture through filtration, centrifugation and so on.

【0025】

Among microbes which produce glucose dehydrogenase, microbes of the genus *Burkholderia*, and *Burkholderia cepacia* in particular is preferably utilized. From *Burkholderia cepacia*, it is possible to obtain the enzyme solution in the form of a solubilized membrane fraction for example. In order to obtain the solubilized membrane fraction, for example, first the microbe is centrifugally separated from a culture, and the microbe is fragmentized to obtain a cell extract. The cell extract is then centrifuged,

and thereafter, obtained supernatant fluid is super-centrifuged. The resulting sediment is the solubilized membrane fraction. The fragmentization of the microbe can be made in an ordinary mechanical or enzymatic method.

**【0026】**

The transformant can be produced by first obtaining DNA which codes manifestation of e.g.  $\alpha$  subunit (a protein having glucose dehydrogenating activity) and  $\beta$  subunit (electron transfer protein), and then introducing the DNA into a host microbe by using a recombinant vector.

**【0027】**

When obtaining the DNA, first, a recombinant vector is constructed. The construction of recombinant vector can be achieved by first separating and purifying a chromosome DNA from a microbe which produces an enzyme having glucose dehydrogenation activity, preparing the chromosome DNA fragments through shredding or PCR amplification, and then binding and closing the chromosome DNA fragments with a linear expression vector.

**【0028】**

Examples of the host microbe include *E. coli* and other enteric bacteria, gram-negative

bacteria such as the genus *pseudomonas* and the genus *Gluconobacter*, gram-positive bacteria including the genus *Bacillus* such as *Bacillus subtilis*, yeasts such as *Saccharomyces cerevisiae*, and filamentous bacteria such as *Aspergillus niger*. Among these, *E. coli* bacteria and those of the genus *pseudomonas* (*pseudomonas putida* for example) are preferably used. The transformation of the microbes can be made by competent cell method through calcium treatment for the genus *Escherichia*. Protoplast methods can be used for the genus *bacillus*, KU method and KUR method for yeasts, and micromanipulation methods for filamentous bacteria. Transformation can also be made by using electroporation method.

#### 【0029】

The enzyme solution is purified by using liquid chromatography. In performing the purification through liquid chromatography, a selection is made for the type, number and combination of different types of chromatography so that a target level of purification can be achieved. Usable types of liquid chromatography include gel filtration chromatography, adsorption chromatography, ion exchange chromatography and affinity chromatography.

**【0030】**

The liquid chromatography may be made by first having the target protein captured in the stationary phase in the column, and then supplying eluent continuously thereby eluting the target protein. Alternatively, a batch method may be used. In the batch method, for example, a column is supplied with a packing agent and a solution which contains a target protein so that the packing agent will hold the target protein. Then, impurities are removed, and eluent is supplied to elute the target protein from the packing agent for collection.

**【0031】**

The eluent is a solution which contains a hydroxy-cholate as an eluting agent. If the liquid chromatography is performed for a plurality of times before yielding the purified target protein, hydroxy-cholate is used as the eluent at least in one cycle of the liquid chromatography. In this case, preferably, hydroxy-cholate is used in the last cycle of liquid chromatography, as the eluent.

**【0032】**

Examples of hydroxy-cholate include cholates, glicoursodeoxycholate, tauroglicoursodeoxycholate,

tauroursodeoxycholate, ursodeoxycholate,  
glycocholate, taurocholate,  
glycochenodeoxycholate, taurochenodeoxycholate,  
glycodeoxycholate, taurodeoxycholate,  
chenodeoxycholate, deoxycholate,  
glycolithocholate, tauroolithocholate and  
lithocholate. Among others, use of cholate,  
such as sodium cholate is preferred.

**【0033】**

When bringing the eluent into contact with the  
packing agent, the concentration of the  
hydroxy-cholate in the solvent may be kept at a  
constant level, or the hydroxy-cholate  
concentration may be linearly changed with the  
time during the supply.

**【0034】**

The liquid chromatography may be made directly  
to the enzyme solution, or may be made after  
the target protein in the enzyme solution has  
been concentrated. The concentration can be  
made by e.g. vacuum concentration, membrane  
concentration, salting-out procedure, factional  
precipitation using hydrophilic solvent (e.g.  
methanol, ethanol and acetone), heating, and  
isoelectric process.

**【0035】**

The purified enzyme thus obtained, is made into a powdery product through such a process as freeze-drying, vacuum-drying and spray-drying, for distribution in the market.

【0036】

【Example】

Hereinafter, specific examples of the fabrication methods outlined above will be described, with demonstration that GDH can be purified more efficiently when the eluent is provided by sodium cholate than when the eluent is provided by NaCl or KCl.

【0037】

<Obtainment of Enzyme Solution>

(1) Obtainment of Enzyme Solution from

Burkholderia Cepacia KS1 Strain

Burkholderia cepacia KS1 strain was cultured under an aerobic condition. More specifically, the KS1 strain was cultured in 20 L of medium at 34°C for 8 hours. The medium contained ingredients listed in Table 1, per liter.



【0038】

【Table 1】

MEDIUM COMPOSITION

Polypeptone	10 g
Yeast extract	1 g
NaCl	5 g
KH <sub>2</sub> PO <sub>4</sub>	2 g
Glucose	5 g
Einol (ABLE Co., Tokyo Japan)	0.14 g
Total, distilled water	1 L
pH	7.2

【0039】

Next, the 20 L of culture medium was centrifuged at 4°C, for 10 minutes at 9000×g, to obtain approximately 250 g of microbe body. The collected microbe body was frozen, then suspended in 10 mM phosphate buffer solution (pH6), and processed in a high-pressure homogenizer (manufactured by Rannie, Denmark) several times at a pressure of 500 bar, to fragmentize cell membrane. The cell extract obtained by cell membrane fragmentation showed a GDH activity of 60 kU. The cell extract was then centrifuged under 8000×g for 60 minutes, to remove cell solid. Further, the supernatant liquid was super-centrifuged at 10°C under 170,000×g for an hour, to collect membrane fraction as the sediment.

【0040】

The membrane fraction was dissipated in 10 mM phosphate buffer solution (pH 6), so as to have a final sodium cholate concentration of 1.5%, and KCl concentration of 0.1 M, and steered at 4°C over a night. As a result, membrane fraction suspension which contained GDH at 30 kU was obtained. The membrane fraction suspension was super-centrifuged at 10°C under 170,000×g for 90 minutes, to remove sediment, and to obtain GDH-containing solubilized membrane fraction at GDH activity of 26kU. The solubilized membrane fraction was dialyzed with 10 mM phosphate buffer solution (pH 6) for 3 nights, and the obtained insoluble matter was removed as sediment in a super centrifugation at 10°C under 170,000×g for 90 minutes. The obtained supernatant liquid (solubilized GDH fraction) contained GDH which showed an activity of 28 kU and a specific activity of 6.8 U/mg.

【0041】

(2) Obtainment of Crude Enzyme Solution from Transformants

First, DNA which includes sequences for coding manifestation of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits was obtained from KS1 strain according to a common method. Next, the obtained DNA was inserted

into plasmid RSF1010 to produce a recombination vector (plasmid RSFGDH  $\gamma\alpha\beta$ ), which was introduced into *Pseudomonas putida* KT2440 strain (ATCC 47054), to make transformants. Each transformant was cultured to obtain the culture solution.

【0042】

The transformant was cultured under normal aerobic conditions, in culture solution including 3.2% polypeptone, 2% yeast extract, 0.5% NaCl, 2% glycerol, 0.05 mL/L Adekanol LG-126 (Asahi Denka Co., Ltd., Tokyo) and 50  $\mu$ g/mL streptomycin (pH 7.0). To 20L of this culture solution, 200 mL of the previous culture solution was inoculated and culture was started at 34°C. In four hours since the beginning of culturing, IPTG (Isopropyl- $\beta$ -D-thiogalactopyranoside) was added at a rate of 0.2 mM, and culturing was continued further for 20 hours, to obtain the target culture solution. This culture solution was centrifuged by a Sharpless centrifuge, and approximately 800 g of microbe body was obtained.

【0043】

Next, the obtained microbe body was suspended in 10 mM phosphate buffer solution (pH 8), fragmented in a high pressure homogenizer (500

bar). Then, Mydol 12 (Kao Corporation, Tokyo) and KCl were added to achieve the final rates of 1% and 0.1 M respectively, followed by steering for 30 minutes. Next, cell solid was separated and removed by centrifuge (8000×g, 60 minutes at 10°C), and supernatant liquid was obtained as crude enzyme solution. The crude enzyme solution was found to have a GDH activity of 2930 kU, and a specific activity of 22 U/mg.

#### 【0044】

##### <Measurement of Glucose Dehydrogenation Activity>

Glucose dehydrogenation activity was measured by tracking a redox reaction of electron acceptor based on glucose dehydrogenation. The electron acceptors were provided by 2,6-dichlorophenol-indophenol (DCIP) and phenazine methosulfate (PMS).

#### 【0045】

Specifically, 900 µL of 47 mM phosphate buffer solution (pH 6.0) containing 20 mM glucose, 2 mM PMS and 0.1 mM DCIP was placed in a spectrophotometer cell, and was pre-incubated at 37°C for 3 minutes. Next, 0.5-10 µL of the enzyme solution was added. The cell was immediately turned upside down to begin

reaction, and time course monitoring was made for absorption drop at a 600 nm wavelength at 37°C.

【0046】

DCIP has an absorption wavelength of 600 nm and the absorption drop is due to a redox reaction of the electron acceptors based on glucose dehydrogenation. Here, calculation of the absorbance was made on the basis that DCIP had a mol absorption coefficient of 4.76 mM/cm. A unit (U) of enzyme was defined as an amount by which 1  $\mu$ M of glucose was oxidized in a minute under the standard measurement conditions. The protein concentration was measured by means of UV method, with a protein concentration defined as being 1g/L for absorption of 1 at 280 nm.

【0047】

Example 1

In this Example, the enzyme solution (solubilized GDH fraction) which was obtained from KS1 strain according to the above-described technique was purified by using a combination of hydrophobic chromatography and anion exchange chromatography.

【0048】

The hydrophobic chromatography was performed by using an Octyl sepharose 4. Fast Flow column (44

mm ID×20 cm Amersham Bioscience KK) which had been equilibrated with 60 mM phosphate buffer solution (pH6). The column was supplied with solubilized GDH fraction which was prepared by adding 1 M phosphate buffer solution (pH 6) to achieve the final concentration of 60 mM. Next, 600 mL of 60 mM phosphate buffer solution (pH 6) and 900 mL of 20 mM phosphate buffer solution (pH 8) were passed, and then tightly adsorbed GDH was eluted by supplying eluent. The eluent was provided by 20 mM phosphate buffer solution (pH 8) which contained sodium cholate in dissolved form. The eluent was supplied at a rate of 15 mL/min so that sodium cholate concentration would vary linearly in the range of 0-1 wt%. As a result, GDH was eluted when the sodium cholate concentration was approximately 0.8 wt%, yielding 340 mL of GDH active fraction. The collected fraction (Octyl collected fraction) had activity readings of 108U/mg specific activity and 16kU of total activity.

【0049】

The anion exchange chromatography was performed by using a Q sepharose Fast Flow column (32 mm ID×12 cm Amersham Bioscience KK) which had been equilibrated with 20mM phosphate buffer

solution (pH 8). To this column, the Octyl collected fraction was supplied, and then 600mL of eluent was supplied. The eluent was provided by 20 mM phosphate buffer solution (pH 8) which contained 1 wt% sodium cholate. The eluent was supplied at a rate of 8 mL/min. As a result, GDH was eluted specifically when approximately 300mL of the eluent had been passed, yielding 340mL of a GDH active fraction. The collected fraction (Q collected fraction) had 770U/mg specific activity, and 14kU total activity.

【0050】

The following Table 2 summarizes the volume of liquid, total activity, specific activity and yield after each operation.

【0051】

【Table 2】

	Liquid Volume	Total Activity	Specific Activity	Yield
Solubilized GDH Fraction	110 mL	28 kU	6.8 U/mg	100%
Octhyl Collected Fraction	340 mL	16 kU	108 U/mg	57%
Q Collected fraction	340 mL	14 kU	770 U/mg	50%

【0052】

### Example 2

In this Example, the crude enzyme solution obtained from the transformant was purified by using a combination of hydrophobic chromatography and anion exchange chromatography.

#### **【0053】**

In the hydrophobic chromatography, first, a Phenyl cellulofine column (300 mm ID×10 cm, Chisso Corporation, Tokyo) which had been equilibrated with 10 mM phosphate buffer solution (pH 8) containing 0.1 M KCl was supplied with the crude enzyme solution, to let the packing agent hold GDH. Next, 7 L of 10 mM phosphate buffer solution (pH 8) containing 0.1 M KCl, and 21L of 10mM phosphate buffer solution (pH 8) were passed. Then, tightly adsorbed GDH was eluted by passing eluent. The eluent was provided by a 10 mM phosphate buffer solution (pH 8) containing sodium cholate in dissolved form. The eluent was supplied at a rate of 7 L/min so that sodium cholate concentration would vary linearly in the range of 0-1 wt%. As a result, GDH was eluted when the sodium cholate concentration was approximately 0.9 wt%, yielding 7300 mL of an active fraction. The collected fraction



(Phenyl collected fraction) had activity readings of 204 U/mg specific activity and 596 kU total activity.

【0054】

The anion exchange chromatography was performed by using a Q sepharose Fast Flow column (44 mm ID×20 cm Amersham Bioscience KK) which had been equilibrated with 10 mM phosphate buffer solution (pH 8). The column was supplied with the Phenyl collected fraction. Note that the phenyl collected fraction was buffer-substituted to 10 mM phosphate buffer solution (pH 8) by using a laboratory module (Asahi Kasei Corporation, Tokyo) which had a 50000 molecular weight cut off, before being supplied to the column. Next, the column was supplied with 600 mL of 10 mM phosphate buffer solution (pH 8), and then with 2,000mL of eluent. The eluent was provided by 10 mM phosphate buffer solution (pH 8) containing 1 wt% sodium cholate. The eluent was supplied at a rate of 10 mL/min. As a result, GDH was eluted specifically when approximately 1400 mL of the 10 mM phosphate buffer solution (pH 8) containing 1 wt% sodium cholate had been passed, yielding 313 mL of a GDH active fraction. The collected fraction (Q

collected fraction) had 1283 U/mg specific activity, and 390 kU total activity.

【0055】

The following Table 3 summarizes the volume of liquid, total activity, specific activity and yield after each operation.

【0056】

【Table 3】

	Liquid Volume	Total Activity	Specific Activity	Yield
Crude Enzyme Solution	1950 mL	2930 kU	22 U/mg	100%
Phenyl Collected Fraction	7300 mL	596 kU	204 U/mg	20%
Q Collected Fraction	315 mL	390 kU	1283 U/mg	13%

【0057】

Further, in this Example, the phenyl collected fraction and the Q collected fraction were subjected to electrophoresis, i.e. SDS-PAGE. The SDS-PAGE used Tris-Tricine buffer solution and was performed in polyacrylamide buffer solution in 8-25% gel gradient. Protein which migrated in the gel was stained with CBB. Results of the SDS-PAGE are shown in Fig. 1(a). In this figure, Lane 2 represents CBB-stained

Phenyl collected fraction and Lane 3 represents CBB-stained Q collected fraction.

【0058】

Example 3

In this Example, the crude enzyme solution obtained from the transformant was purified by using a combination of hydrophobic chromatography and anion exchange chromatography.

【0059】

The hydrophobic chromatography was performed in the same way as in Example 2. The collected solution was ultracondensed, to yield 70 mL of phenyl collected fraction, which had a specific activity of 300 U/mg and a total activity of 21 kU.

【0060】

The anion exchange chromatography was performed by using a QAE-TOYOPEARL 550 column (44 mm ID×10 cm, Tohso Corporation, Tokyo) which had been equilibrated with 10 mM phosphate buffer solution (pH 8). The column was supplied with the phenyl collected fraction, and then with 2,000 mL of eluent. The eluent was provided by 10 mM phosphate buffer solution (pH 8) containing 1 wt% sodium cholate. The eluent was supplied at a rate of 5 mL/min. As a

result, GDH was eluted specifically when approximately 1600 mL of the eluent has been passed, yielding 300 mL of active fraction. The collected fraction (QAE collected fraction) had 1500 U/mg specific activity, and 7.8 kU total activity.

【0061】

The following Table 4 summarizes the volume of liquid, total activity, specific activity and yield after each operation.

【0062】

【Table 4】

	Liquid Volume	Total Activity	Specific Activity	Yield
Crude Enzyme Solution	420 ml	80 kU	59 U/mg	100%
Phenyl Collected Fraction	70 mL	21 kU	300 U/mg	26%
QAE Collected Fraction	300 mL	7.8 kU	1500 U/mg	10%

【0063】

Further, in this Example, the QAE collected fraction was subjected to SDS-PAGE, using the same procedure as used in Example 2, and the protein was stained with CBB. Results of the SDS-PAGE are shown in Fig. 1(a). In this

figure, Lane 4 represents CBB-stained QAE collected fraction.

【0064】

Comparative Example 1

In this Comparative Example, the crude enzyme solution obtained from the transformant was purified by using a combination of hydrophobic chromatography and anion exchange chromatography.

【0065】

The hydrophobic chromatography was performed in the same way as in Example 2, yielding 7200 mL of phenyl collected fraction, which had specific activity of 314 U/mg and total activity of 256 kU. In this Comparative Example, however, a portion of the phenyl collected fraction or 1100 mL (Q apply) which represents a total activity of 39 kU was subjected to the following anion exchange chromatography.

【0066】

The anion exchange chromatography was performed by using a Q sepharose Fast Flow column (44 mm ID×13 cm Amersham Bioscience KK) which had been equilibrated with 10 mM phosphate buffer solution (pH 8). The column was supplied with the Q apply. Thereafter, the column was

supplied with 800 mL of 10 mM phosphate buffer solution (pH 8) in order to remove non-adsorbent protein. Then, the column was supplied with eluent. The eluent was provided by 10 mM phosphate buffer solution (pH 8) which contained NaCl in dissolved form. The eluent was supplied at a rate of 7.5 L/min so that NaCl concentration would vary linearly in the range of 0-0.6 M. As a result, GDH was eluted at two NaCl concentration levels of 0.25 M approx. and 0.4 M approx., yielding 140 mL and 360 mL of active fractions respectively. Each of the active fractions (Q collected fraction (1) and Q collected fraction (2)) were subjected to activity measurement. The Q collected fraction (1) had specific activity of 600 U/mg and total activity of 4.5 kU. The Q collected fraction (2) had specific activity of 432 U/mg and total activity of 12 kU.

**【0067】**

The following Table 5 summarizes the volume of liquid, total activity, specific activity and yield after each operation.

【0068】

【Table 5】

	Liqu id Volu me	Total Activi ty	Specif ic Activi ty	Yie ld
Crude Enzyme Solution	1250 mL	1066 kU	36 U/mg	100 %
Phenyl Collected Fraction	7200 mL	256 kU	314 U/mg	24%
Q Apply	1100 mL	39 kU	314 U/mg	24%
Q Collected Fraction (1)	140 mL	4.5 kU	600 U/mg	3%
Q Collected Fraction (2)	360 mL	12 kU	432 U/mg	7%

【0069】

Further, in this Comparative Example, the phenyl collected fraction and the Q collected fraction (2) were subjected to SDS-PAGE using the same procedure as used in Example 2, and the protein was stained with CBB. Results of the SDS-PAGE are shown in Fig. 1(b). In this figure, Lane 2 represents the phenyl collected fraction and Lane 3 represents the Q collected fraction (1).

【0070】

Comparative Example 2

In this Comparative Example, a portion of the phenyl collected fraction obtained in Comparative Example, or the 2100 mL (QAE apply)

which represents the total activity of 74kU, was subjected to anion exchange chromatography, to obtain purified GDH.

**【0071】**

The anion exchange chromatography was performed by using a QAE-TOYOPEARL 550 column (44 mm ID×10 cm Tohso Corporation, Tokyo) which had been equilibrated with 10 mM phosphate buffer solution (pH 8). The column was first supplied with the QAE collected fraction (74 kU total activity). Next, 800 mL of 10 mM phosphate buffer solution (pH 8) was supplied to remove non-adsorbent protein. Then, the column was supplied with eluent. The eluent was provided by 10 mM phosphate buffer solution (pH 8) containing dissolved KCl. The eluent was supplied at a rate of 5 mL/min so that KCl concentration would vary linearly in the range of 0-1M. As a result, GDH was eluted at two KCl concentration levels of 0.23 M approx. and 0.43 M approx., yielding 200 mL and 400 mL of active fractions respectively. Each of the active fractions (QAE collected fraction (1) and QAE collected fraction (2)) were subjected to activity measurement. The QAE collected fraction (1) had specific activity of 399 U/mg and total activity of 7.4 kU. The QAE



collected fraction (2) had specific activity of 217 U/mg and total activity of 6.4 kU.

【0072】

The following Table 6 summarizes the volume of liquid, total activity, specific activity and yield after each operation.

【0073】

【Table 6】

	Liquid Volume	Total Activity	Specific Activity	Yield
Crude Enzyme Solution	1250 mL	1066 kU	36 U/mg	100%
Phenyl Collected Fraction	7200 mL	256 kU	314 U/mg	24%
QAE Apply	2100 mL	74 kU	314 U/mg	24%
QAE Collected Fraction (1)	200 mL	7.4 kU	399 U/mg	2%
QAE Collected Fraction (2)	400 mL	6.4 kU	217 U/mg	2%

【0074】

Further, in this Comparative Example, the QAE collected fraction was subjected to SDS-PAGE using the same procedure as used in Example 2, and the protein was stained with CBB. Results of the SDS-PAGE are shown in Fig. 1(b). In this figure, Lane 4 represents the QAE collected fraction (2).

【0075】

<Discussion on the Results>

As understood from Table 2 through Table 6, GDH has a higher final specific activity and is purified more efficiently when the eluent is provided by cholate (Example 1 through 3) than when the eluent is provided by NaCl or KCl and GDH is purified through gradient elution (Comparative Example 1 and 2). This is also indicated in Fig. 1(a) and Fig. 1(b). Specifically, GDH derived from the KS1 strain includes  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, and their molecular weights in the SDS gel-electrophoretic migration under reducing environment were approximately 60 kDa, 43 kDa and 14 kDa respectively. With this point in mind, Fig. 1(a) and Fig. 1(b) will reveal that the collected fractions in Example 2 and Example 3 have wider bands which represent  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, than the corresponding bands found in the collected fractions in Comparative Examples 1 and 2, while having smaller portions of proteins which have different molecular weights. Therefore, when purifying GDH by using a combination of hydrophobic chromatography and anion exchange chromatography, use of sodium cholate for eluting GDH will lead to efficient purification of GDH.

**【Brief Description of the Drawings】**

**【Fig. 1】** shows an SDS-PAGE result of an enzyme which was purified with anion exchange chromatography. (a) shows a result of an enzyme using a sodium cholate eluent. (b) shows a result of an enzyme using a NaCl or KCl eluent.

**【Identification of the Document】** ABSTRACT

**【Abstract】**

**【Object】**

The present invention relates to a method for purifying target protein.

**【Means】**

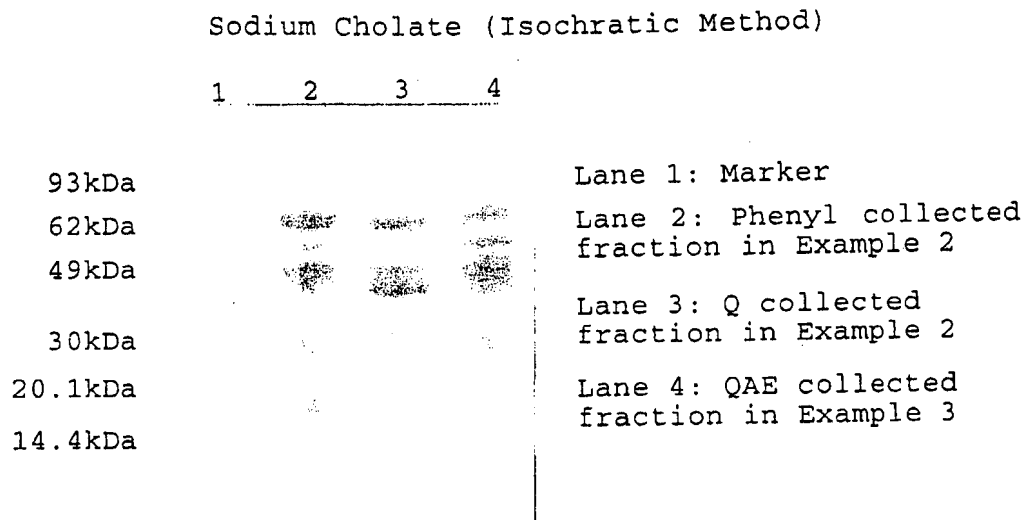
A method for purifying a target protein from a protein solution containing the target protein including electron transfer protein uses liquid chromatography. The liquid chromatography is performed by introducing the protein solution into a column filled with a packing agent, causing the packing agent to hold the target protein, removing impurities, eluting the target protein in the packing agent by using an eluent containing a hydroxy-cholate.

**【Selected Figure】** None

【Identification of Document】

Drawings

【Fig. 1】



【Fig. 2】

